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The physiological effects of the seed treatment Stamina on maize seedling length, ADP:O ratios, and respiration rates

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by

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ABSTRACT

Zea mays L. is an important crop in the Midwest, and each year there is a push to plant earlier in the spring. Early planting can leave seeds and seedlings vulnerable to low temperature stress. Low temperature stress causes damage to maize seedlings. It has been postulated that Strobilurin fungicides have positive physiological related effects on growth and yield. The goal of this study was to determine if the strobilurin Stamina protects maize seedlings against mitochondrial damage due to low temperature stress. In this study, three seed treatments and two seed lots/hybrids were used. Treatments included: Stamina (binders plus active ingredients), binders only, and control (untreated) seedlings. To determine the effects of Stamina on maize respiration, seedling lengths, ADP:O ratios, respiration rates, and alternative oxidase (appendix) were measured under optimal temperature and low temperature conditions in the presence and absence of the alternative oxidase inhibitor salicylhydroxamic acid (SHAM). Seedlings were grown in dark growth chambers at a constant 25°C for 5 days or for 10 days at 10°C and 4 days at 25°C. In the optimal temperature study, seedlings from Stamina and only binders treated seeds had mesocotyl lengths that were 0.48 cm and 0.41 cm longer than control seeds, respectively. Seedlings from Stamina treated seeds had total lengths that were 0.38 cm and 0.87 cm longer than seeds treated with binders only and control seedlings, respectively. Stamina had no effect on ADP:O ratios, AOX ratios, respiration rates, or respiration rate ratios. In the low temperature stress study, there was a hybrid by treatment interaction. Stamina did not affect seedling length of seed lot/hybrid A, but it did of seed lot/hybrid B. Seedlings from Stamina treated seeds of seed lot/hybrid B had mesocotyl lengths that were 0.9 cm longer than both seedlings treated with only binders

and control seedlings. Seedlings from Stamina treated seeds of the same seed lot/hybrid had total lengths that were 1.16 cm and 1.21 cm longer than seedlings treated with only binders and control seedlings, respectively. Seedlings from Stamina treated seeds and control seedlings had AOX ratios that were slightly larger than AOX ratios for seedlings treated with only binder when 20 mM SHAM was used. Seedlings from Stamina treated seeds and control seedling of seed lot/hybrid A had slightly larger respiration rate ratios than seedlings from seeds treated with only binders when 10 mM SHAM was used. Seedlings from Stamina treated seeds had slightly larger respiration rate ratios than control seedlings of seed lot/hybrid B when 10 mM SHAM was used.

CHAPTER ONE – LITERATURE REVIEW

Low Temperature Stress

Soil temperature and field conditions usually determine when maize is planted in the spring in the Midwest. Each year producers push to plant maize earlier in the spring to take advantage of the entire growing season. However, with early planting comes the risk of low temperature stress injury to seeds and germinating seedlings (Lauer et al., 1999).

Low temperatures can impact seedling performance in many ways depending on the actual temperature experienced and the duration of exposure. The optimum temperature range for maize seedling germination and growth is 25-30°C (Greaves, 1996; Prasad et al., 1994a; Prasad et al., 1994b; Stewart et al., 1990a). Deviation from this range for extended periods of time can alter seedling growth. As temperatures fall below 20°C, seedling growth can be inhibited. Temperatures below 10°C can eventually lead to tissue and cellular damage (Greaves, 1996; Stewart et al., 1990a).

Respiration is a critical process for plants, especially during germination and emergence. It has been demonstrated that maize seedling mitochondria are susceptible to low temperature stress (Prasad et al., 1994a). Low temperature stress can reduce O_2 uptake, electron flow through the cytochrome pathway, and ATP production. This leads to an overall decrease in mitochondrial respiration (Prasad et al., 1994a). When the mitochondrial electron transport chain (ETC) is inhibited or malfunctioning, reactive oxygen species (ROS) formation in the ETC can increase (Purvis & Shewfelt, 1993). Some ROS induce lipid peroxidation that can damage membranes (Fridovich, 1978). This leads, at least in part, to the visible signs of low temperature stress such as: inhibited

seedling growth, browning and desiccation of the seedlings, and death (Prasad et al., 1994b). The effect of low temperature stress on plant respiration will continue to be an important area of investigation as producers push to plant earlier.

Respiration

Respiration involves three metabolic pathways: Glycolysis, the Citric Acid Cycle, and the Electron Transport Chain (ETC), which supports Oxidative phosphorylation. Glycolysis, located in the cytosol and plastids, oxidizes sugars and starches to pyruvate. In the mitochondrial matrix, pyruvate is oxidized through the Citric Acid Cycle to produce carbon skeletons and reducing equivalents such as NADH and $FADH_2$. Oxidative phosphorylation occurs in the inner membrane of the mitochondria and uses the mitochondrial ETC to transfer electrons from the products of the Citric Acid Cycle (NADH and $FADH_2$) to oxygen to produce water. The transfer of electrons through the electron transport system creates a proton electrochemical gradient across the inner mitochondrial membrane that is used to drive ATP synthesis (van Dongen et al., 2011).

There are 5 states of respiration in isolated mitochondria (Gnaiger, 2010). When mitochondria are first added to the system in the absence of a substrate, the mitochondria are in State I respiration. State II respiration is initiated with the addition of a substrate such as: NADH, succinate, or malate (Ikuma & Bonner, 1967). State II respiration occurs at a low rate due to the lack of ADP and P_i. State III respiration occurs when ADP and P_i are added to the system. The binding of ADP and P_i to ATP Synthase causes protons to be transferred to the matrix from the inner membrane space, driving the formation of ATP. When all the ADP and P_i added to the system has been converted to ATP and the

respiration rate slows, the mitochondria are in State IV respiration. State V respiration is the depletion of oxygen from the system (Gnaiger, 2010).

Electron Transport Chain

The mitochondrial ETC is composed of four multi-protein complexes (I, II, III, and IV) and two mobile carriers that transfer electrons from the reducing equivalents produced in the Citric Acid Cycle to molecular oxygen to form water (Figure 1). Complexes I, III, and IV pump protons from the mitochondrial matrix to the inner membrane space as electrons flow through these complexes. This proton pumping is responsible for the creation of a proton electro-chemical gradient across the inner mitochondrial membrane. The proton gradient drives the production of ATP via the F_oF_1 – ATPase (Complex V). The ETC also contains several non-phosphorylating complexes such as: internal and external NAD(P)H dehydrogenases, electron transfer flavoprotein, electron transfer flavoprotein quinone oxidoreductase, glycerol-3-phosphate dehydrogenase, alternative oxidase, and uncoupling proteins (Moore & Siedow, 1991; van Dongen et al., 2011).

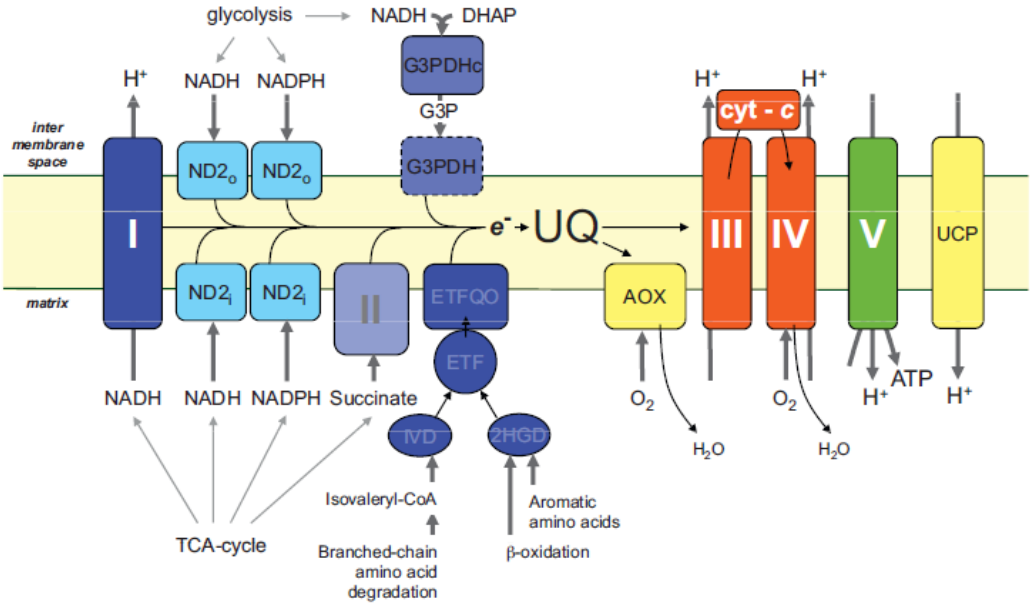

Figure 1: Components of the mitochondria ETC (Reprinted from the Journal of Plant Physiology, 168(12), van Dongen, J.T., Gupta, K.J., Ramirez-Aguilar, S.J., Araujo, W.L., Nunes-Nesi, A., &Fernie, A.R, Regulation of Respiration in plants: a role for alternative metabolic pathway, 1436. Copyright (2011), with permission by Elsevier)

Complex I

The ETC starts with Complex I, also known as the NADH dehydrogenase. NADH dehydrogenase transfers electrons from reducing equivalents produced in the Citric Acid cycle (NADH) to the Ubiquinone. This process translocates a proton from the matrix to the inner membrane space, contributing to the electrochemical proton gradient (Millar et al., 2011). Complex I is also a location for possible superoxide generation (Purvis & Shewfelt, 1993).

Complex II

Complex II is referred to as succinate dehydrogenase. This complex also transfers electrons from Citric Acid Cycle products (succinate) to Ubiquinone. Succinate

dehydrogenase, however, is not a membrane spanning protein like NADH dehydrogenase and does not participate in proton pumping across the membrane. Complex II is the only complex that is part of the Citric Acid Cycle and the ETC (Millar et al., 2011).

Ubiquinone

Ubiquinone is a small lipid mobile carrier that transfers electrons from the dehydrogenases to the oxidases (Millar et al., 2011). The Cytochrome oxidase pathway and the alternative oxidase pathway oxidizes the reduced ubiquinone pool to produce water (Moore & Siedow, 1991).

Complex III

Complex III is called cytochrome c reductase. Cytochrome c reductase receives electrons from the ubiquinone pool and transfers those electrons to Complex IV via cytochrome c. Complex III also participates in proton pumping (van Dongen et al., 2011). Complex III, specifically the Q_0 site, is involved in the production of ROS (Bleier & Drose, 2013; Purvis & Shewfelt, 1993).

The manner in which Complex III transfers electrons from the ubiquinone pool to cytochrome c is called the Q cycle. Complex III contains an ubiquinol oxidation center (Q_0), a ubiquinone reduction center (Q_i), and several prosthetic groups: cytochrome b, cytochrome c_1, and the Rieske protein. To start the cycle, ubiquinol is oxidized to ubiquinone at the Q_0 site of Complex III. One electron is fed to the Rieske protein, which causes the Rieske protein head to move from a location close to cytochrome b to a position close to cytochrome c_1. The electron is transferred to cytochrome c. As this transfer of electrons takes place, protons are pumped from the matrix to the inner

membrane space. The second electron, which is transported onto ubiquinone bound to the Q_i site, is used to form a stable semiquinone. To complete the cycle, the process is repeated (Bleier & Drose, 2013).

Cytochrome C

Cytochrome C is a small protein that acts as mobile carrier that transfers electrons from Complex III to Complex IV (Millar et al., 2011; Moore & Siedow, 1991).

Complex IV

Complex IV, the terminal oxidase, is known as cytochrome c oxidase. Cytochrome c oxidase receives electrons from cytochrome c reductase via the cytochrome c mobile carrier and pumps protons from the matrix to the inner membrane space (Millar et al., 2011; Moore & Siedow, 1991). This complex reduces oxygen to form water (van Dongen et al., 2011)

Complex V

Although not technically part of the ETC, the F_0F_1 – ATP synthase complex is referred to as Complex V (Siedow & Umbach, 1995). This complex uses the proton electrochemical gradient produced by Complexes I, III, and IV to produce ATP from ADP and P_i (van Dongen et al., 2011). It has been demonstrated that under low temperature stress, the activity of complex V can decrease by 30-35% (Prasad et al., 1994a).

The F_1 complex, which contains α, β, γ, δ, and ε subunits, is attached to the membrane bound F_0 complex. Subunits α and β are arranged in a circular orientation

creating a catalytic complex. The γ subunit, which is attached to the F_0 complex, is inserted through the middle of catalytic complex. The subunit δ attaches the β subunits of the F_1 complex to the inner membrane. During ATP synthesis, protons move through the F_0 complex from the inner membrane space toward the matrix causing the complex to rotate. This rotation causes the γ subunit of the F_1 complex to rotate. Rotation of this subunit leads to conformational changes within the catalytic complex. Within this catalytic complex, there are three conformations in the β subunits: loose, open, and tight. The substrates for ATP synthesis, ADP and P_i, bind to the open site. The rotation of γ subunit changes the conformation from open, to loose, to tight. ATP is synthesized in the tight conformation and released in the open conformation (Duncan et al., 1995; Junge et al., 1997).

Non – phosphorylating complexes

Type II NAD(P)H dehydrogenases (ND2) are non-membrane spanning proteins that use NADH and NADPH from the Citric Acid Cycle to reduce ubiquinone. The participation of this complex in the ETC can change depending on physiological conditions and stress. Co-expression of ND2 and alternative oxidase may play a role in ROS production regulation (van Dongen et al., 2011).

Plant uncoupling proteins (UCP) are activated by free fatty acids and transport protons from the inner membrane space to the mitochondria matrix causing a decrease in the proton electrochemical gradient. This leads to a decrease in ATP production. It is thought increased UCP activity can protect membranes from ROS. ROS can cause lipid peroxidation which leads to the production of 4-hydroxy-2-trans-nonenal. This product activates UCP, which in turn limits ROS production under stress. UCPs may also aide in

the synthesis of intermediates of amino acids and lipid biosynthesis. Unlike alternative oxidase, it is thought that UCPs are involved in prolonged mediation of energy balance (van Dongen et al., 2011).

The electron transfer flavoprotein and electron transfer flavoprotein quinone oxidoreductase make up the ETF/EFTQO complex. This complex, which is induced by oxidative stress, is involved in the oxidation of fatty acids and amino acids to provide an alternative source of electrons form the ETC (van Dongen et al., 2011).

The glycerol – 3 – phosphate dehydrogenase cycle involves the transfer of electrons from cytosolic NADH to the ubiquinone via a cytosolic NAD^+-dependent G3P dehydrogenase linked to a mitochondrial FAD-dependent G3P dehydrogenase. During stress, this pathway may be involved in regulation of ROS production (van Dongen et al., 2011).

Alternative Oxidase

One of the non-phosphorylating complexes that has been studied for several years is alternative oxidase. Alternative oxidase, a second terminal oxidase in the ETC of fungi, algae, protozoa, and plants, was discovered to be insensitive to traditional cytochrome oxidase inhibitors such as: cyanide, azide, and carbon monoxide and sensitive to salicylhydroxamic acid (SHAM) and n-propyl gallate (Bendall & Bonner, 1971; Siedow & Umbach, 1995). Alternative oxidase branches from the cytochrome oxidase pathway near ubiquinone between Complex II and Complex III (Bendall & Bonner, 1971; Storey, 1976). Complexes III and IV are bypassed when electrons flow through alternative oxidase, leading to a reduction in electrochemical gradient and the amount of ATP

generated. Like cytochrome oxidase, electrons are transferred to molecular oxygen to form water (Siedow & Umbach, 1995).

The role of alternative oxidase is best known in thermogenic plant species. Thermogenic plants produce heat when electrons are diverted through alternative oxidase. This heat production aids in pollination by ensuring that it is warm enough for pollination to occur and by volatilizing compounds that will attract pollinators (Meeuse, 1975). The role of alternative oxidase in non thermogenic plants is not as clear. It was thought that alternative oxidase was only engaged as an electron overflow mechanism when the cytochrome oxidase was saturated (Lambers, 1982). However, it has been demonstrated that electrons are distributed between alternative oxidase and cytochrome oxidase and that neither pathway is fully engaged under normal conditions with one substrate (Elthon et al., 1986; Hoefnagel et al., 1995; Prasad et al., 1994a). The distribution of electrons between alternative oxidase and cytochrome oxidase depends in part on the redox state of the ubiquinone pool (Dry et al., 1989; Ribas-Carbo et al., 1995). Alternative oxidase is now thought to provide short term protection against oxidative damage due to changes in respiration activity (Maxwell et al., 1999; van Dongen et al., 2011) and allow for respiration to continue when it would otherwise be restricted (Maxwell et al., 1999; Rasmusson et al., 2009; Stewart et al., 1990a; van Dongen et al., 2011).

As stated earlier, low temperature stress can lead to the production of ROS in the mitochondrial ETC which leads to oxidative stress. The production of ROS is thought to be involved in the up-regulation of alternative oxidase. An increase in alternative oxidase activity decreases ROS production to protect the cell against oxidative damage (Skutnik

& Rychter, 2009). The presence of ROS can induce the expression of AOX genes, specifically AOX1a which is highly associated with low temperature stress in maize (Clifton et al., 2005; Considine et al., 2002). Currently, monocots are known to possess only the AOX1 genes (Considine et al., 2002).

It has been shown that the mesocotyl of maize seedlings express alternative oxidase activity at optimum temperatures and under low temperature stress. With a decrease in temperature, there was an increase in alternative oxidase. Seedlings that grew best under cold temperature stress had the highest alternative oxidase activity. Under optimum temperatures, alternative oxidase activity can account for about 23% of mitochondria respiration and cytochrome oxidase accounts for about 62% of mitochondria respiration. Under low temperature stress, it was shown that alternative oxidase activity can increase to 31-43% of oxygen uptake. Whereas cytochrome oxidase activity decreases to around 30% of oxygen uptake (Elthon et al., 1986; Prasad et al., 1994a; Stewart et al., 1990a).

ADP:O ratios

ADP:O ratios measure the amount of ADP phosphorylated to ATP per nmol of oxygen consumed (Estabrook, 1967). ADP:O ratios are substrate dependent and are about 75% of theoretical values (Ikuma & Bonner, 1967). Because there are three complexes that pump protons into the intermembrane space, the theoretical highest ADP:O ratio is around 3. For succinate, the highest ADP:O ratio is around 2 (Ikuma & Bonner, 1967). With succinate as a substrate electrons enter the ETC through complex II, which does not participate in proton pumping (Miller et al., 2011). A good ADP:O ratio for non-

thermogenic species, under normal temperature conditions, is 1.35 to 1.6 with succinate as a substrate (Hoefnagel et al., 1995; Ikuma & Bonner, 1967; Lyons & Raison, 1970).

Strobilurins and Pyraclostrobins

Strobilurins are synthetic agricultural fungicides used as foliar applications, seed treatment applications, and in-furrow applications on cereal, fruit, vegetable, and nut crops (Bartlett et al., 2002). These synthetic fungicides are modeled from strobilurin A, which is produced by the Basidiomycete *Strobilurus tenacellus* (Anke et al., 1977). Strobilurins are effective against Ascomycetes, Basidiomycetes, Deuteromycetes, and Oomycestes (Bartlett et al., 2002; Munkvold, 2009).

Strobilurins bind at the Q_0 site of cytochrome b in the cytochrome bc_1 complex (Complex III) of the mitochondrial ETC (Anke, 1995). Binding at this site prevents electron flow from cytochrome b to cytochrome c, which will ultimately decrease mitochondrial respiration (Becker et al., 1981; Sauter et al., 1995). Due to their inhibitory nature, there were concerns that strobilurins could be toxic to other eukaryotes (Sauter et al., 1995). Roehl & Sauter (1993) found that strobilurins, specifically kresoxim-methyl, inhibited electron flow in maize. However, maize had a lower sensitivity to strobilurins compared to other eukaryotes, indicating they could be used as an agricultural fungicide (Roehl & Sauter, 1993).

It has been demonstrated that for some strobilurins, some fungal species were able to survive respiration inhibition by up-regulating alternative oxidase (Olaya et al., 1998). Survival of inhibition due to alternative oxidase is more prevalent at later stages of fungal

development (mycelial stage). However there are instances of alternative oxidase during spore germination (Olaya et al., 1998).

According to Mizutani et al (1998), alternative oxidase is up regulated in rice roots after incubation with SSF 126. SSF 126 is a methoxyiminoacetamide systemic fungicide that, like strobilurins, inhibits electron flow through the ETC by inhibiting electron flow through the complex III (Mizutani et al., 1998).

It is believed that Strobilurins can have positive physiological related benefits such as improved stands and yield, especially under adverse environmental conditions (Gerhard et al., 1998; Munkvold, 2009). Strobilurins have been found to increase soil nitrogen uptake, improve nitrogen use efficiency, increase the duration of green leaves, decrease ethylene production, and decrease the CO_2 compensation point in wheat (Gerhard et al., 1998; Grossmann & Retzlaff, 1997; Ishikawa et al., 2011)

BASF Corporation developed the strobilurin, pyraclostrobin (Stamina). What distinguishes pyraclostrobin from other strobilurins, is that pyraclostrobin has a methyl N-methoxycarbamate toxophore. Pyraclostrobins are effective against spore germination, mycelial growth, and some spore production on diseased leaves. (Bartlett et al., 2002; Karadimos et al., 2005).

Project Goals

Maize is an important crop in the Midwest, and each year there is a push to plant earlier in the spring. Early planting can leave seeds and seedlings vulnerable to low temperature stress. Low temperature stress causes damage to maize seedlings. Strobilurin fungicides are believed to have positive, physiological related effects on growth and

development. The goal of this study was to determine if the strobilurin Stamina protects maize seedlings against oxidative damage due to low temperature stress. Mesocotyl and total seedling lengths, ADP:O ratios, respiration rates, and alternative oxidase (appendix) were measured at optimal temperature conditions and low temperature stress conditions to determine to effects of Stamina on mitochondrial respiration.

CHAPTER TWO – MATERIALS AND METHODS

Plant Material

Treated seeds were provided by BASF. Seed treatments included: water treated (no treatment), formulation blank (binders only), and formulated (binders plus active ingredients). Seeds came labeled as treatment 1 (no treatment), 2 (binders only), or 3 (binders plus active ingredients). The four maize seed lots/hybrids, labeled: 1, 2, 3, and 4 were supplied by BASF. Seeds provided were referred to as seed lot/hybrid due to a lack of multiple seed lots per hybrid. Two seed lot/hybrids were selected for this study based on growth appearance in germination paper. To determine which seed lots/hybrids were used, 50 untreated seeds from each seed lot/hybrid were grown on germination paper for 5 days at 25° C. Hybrids 2 (A) and 3 (B) were selected because they looked healthier, grew better, and had fewer pathogens compared to hybrids 1 and 4.

Growth Conditions

Two separate studies were conducted. The first study was conducted at optimal temperature conditions and the second included a low temperature stress period. In both studies, seedlings were grown in the dark. For each experimental observation 11 germination paper rolls consisting of 25 seeds each were prepared. Germination paper rolls were randomly placed in germination buckets. Plastic bags were secured over the buckets and the buckets were randomly placed in the growth chambers. Seedlings were grown for 5 days at 25° C for the first study. For the second study, seedlings were grown in the growth chamber for 10 days at 10° C and 4 days at 25° C. The second study was originally based on a previous temperature stress treatment of 10 days at 10 °C, 4 days at

25 °C and 10 days at 10 °C done in our lab (Hejlik, 2012). However, functional mitochondria could not be isolated after the second temperature stress period.

Seedling Length Measurements

Both seedling mesocotyl and total seedling lengths were measured. Seedling mesocotyls were measured from the scutellar joint to the coleoptile (Raju & Steeves, 1998). Total seedling length was measured from the scutellar joint to the tip of the coleoptile. Seedling length measurements were taken from three randomly selected germination paper towel rolls per each experimental observation.

Mitochondria Extraction

The protocol for mitochondria extraction was modeled from a previous protocol developed in our lab based on Heckman et al. (2002) and Prasad et al. (1994a) and several other sources (Estabrook, 1967; Millar et al., 2007; Neuburger et al., 1982). Mesocotyl tissue (45 grams) was harvested using a scalpel and weighed for extraction. Mesocotyl tissue was placed in a plastic bag in the refrigerator (~5°C) until grinding. The mesocotyl tissue, 150 mL of chilled grinding medium (350 mM Mannitol, 30 mM MOPS, 1mM EDTA, 0.5 % BSA, pH 7.6), 1 g PVP, and 0.2 g L-cysteine were added to a chilled Waring blender. The mixture was ground for 3 x 5 second bursts on the high setting. The ground tissue was filtered through two layers of cheesecloth into chilled beakers. Equal aliquots of the mixture were poured into 4 x 50 mL conical bottom centrifuge tubes. Balanced centrifuge tubes were centrifuged in a Sorvall Evolution RC centrifuge and Sorvall SLA – 600TC Super-Light rotor for 4 minutes at 5,200 g at 2° C. The resultant supernatant was transferred to fresh 50 mL conical bottom tubes and

centrifuged for 5 minutes at 19,300 g at 2° C. The supernatant was removed and the resultant pellet was rinsed with 1 mL of chilled wash medium (300 mM Mannitol, 20 mM MOPS, 1 mM EDTA, 0.5% BSA, pH 7.2). The washed pellet was re-suspended in 1 mL of chilled wash medium. The re-suspended pellet was layered on top of 28 mL of chilled 28% Percoll gradient in round bottom high speed centrifuge tubes. The Percoll gradient consists of 3 components: Percoll, 2.5 M sucrose, and solution A (300 mM Sucrose, 10 mM Potassium Phosphate, 1 mM EDTA, 0.1% BSA, pH 7.2). The gradient was made by making a solution of a 9:1 Percoll and 2.5 M sucrose with solution A. Tubes were centrifuged for one hour at 40,000 g at 2° C in a Thermo Scientific Fiberlite F 21-8x50y rotor. During centrifugation, a distinct band of purified mitochondria forms in the lower half of the tube (Figure 2). It was determined using density marker beads (Cospheric^{TM} , Santa Barbara, CA) that the band formed at a density of 1.09 g/cc, which is similar to with densities measured by Moreu & Romani (1982) and Neuburger et al. (1982). The band of mitochondria was removed with a pipette and placed in a new 50 mL conical bottom centrifuge tube. 35 mL of chilled Percoll wash medium (300 mM Mannitol, 1 mM EDTA, 10 mM Potassium Phosphate, 0.1% BSA, pH 7.2) was added to each centrifuge tube. Centrifuge tubes were centrifuged for 15 minutes at 12,000 g at 2° C. The result of this centrifugation was a brown pellet of purified mitochondria at the bottom of the tube. The pellet was removed with a pipette and placed in a microfuge tube. The pellet was suspended in 650 µL of suspension medium (250 mM Sucrose, 30 mM MOPS, pH 7.2). The microfuge tubes were placed in the -80° freezer until use for the protein assays.

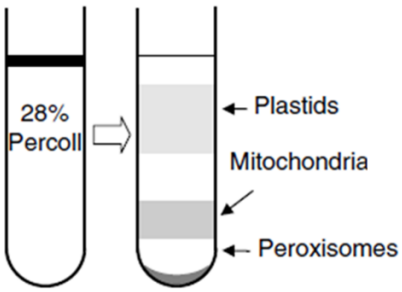

Figure 2: The layering of plant organelles after centrifugation with the 28% Percoll gradient. (Adapted from Methods in Cell Biology, 80, Miller, A.H., Liddell, A., & Leaver, C.J., Isolation and Subfractionation of mitochondria from plants, 70. Copyright (2007), with permission from Elsevier)

Respiration Rates and ADP:O Ratios

The protocol for measuring respiration rates and ADP:O ratios was modeled from a previous protocol developed in our lab based on Heckman et al. (2002) and Prasad et al. (1994a) and other sources (Stewart et al., 1990a; Stewart et al., 1990b). The mitochondria activity was assessed using an oxytherm system and Hansatech Oxygen Electrode (Clark electrode) from Hansatech Instruments. The oxytherm system was set up according to the set up instruction (Hansatech Instruments). Once the system was ready for samples, 1.5 mL of reaction medium (250 mM Sucrose, 30 mM MOPS, 5 mM Potassium phosphate, 5 mM Magnesium Chloride, 0.5 % BSA, pH 7.2) was added to the reaction vessel. When the rate steadied, 500 µL of chilled mitochondrial sample was added to the reaction vessel. It was important that the mitochondria samples remained chilled until they were added to the oxytherm system. Prolonged exposure to room temperature reduced the quality of the mitochondria. After the rate steadied, 10 µL of 100 mM succinate was added as the substrate. Once the rate steadied, 10 µL of 10 mM ADP and P_i was added. The addition of ADP and P_i induced a cycle of phosphorylation to make ATP. The rate

changes between State IV respiration and State III respiration could be seen in the oxytherm output. This output was used to calculate the ADP:O ratios which were used determine the activity and quality of the mitochondria. Once the rate steadied again, additional ADP and P_i was added.

Because alternative oxidase is sensitive to SHAM, SHAM was used to inhibit electron flow through alternative oxidase. After the third addition of ADP and P_i, 10 µL of 10 mM SHAM was added at the end of the phosphorylation cycle. In the optimal temperature experiment, 10 µL of 10 mM SHAM was added at the end of the 3rd and 4th phosphorylation cycles, and 10 µL of 20 mM SHAM was added at end of 5th and 6th phosphorylation cycles. In the low temperature stress experiment, 10 µL of 10 mM SHAM was added at the end of the 3rd phosphorylation cycle and 10 µL of 20 mM SHAM was added at the end of the 4th phosphorylation cycle. In the optimal temperature experiment, oxygen was sometimes depleted before the 5th and 6th phosphorylation cycles. Due to varied depletion of oxygen before all six phosphorylation cycles in the optimal temperature stress experiment, in the low temperature stress experiment the 10 µL of 20 mM SHAM was added earlier to determine if a higher concentration would have a different impact. An example of oxytherm output with a mitochondria sample is provided below in Figure 3.

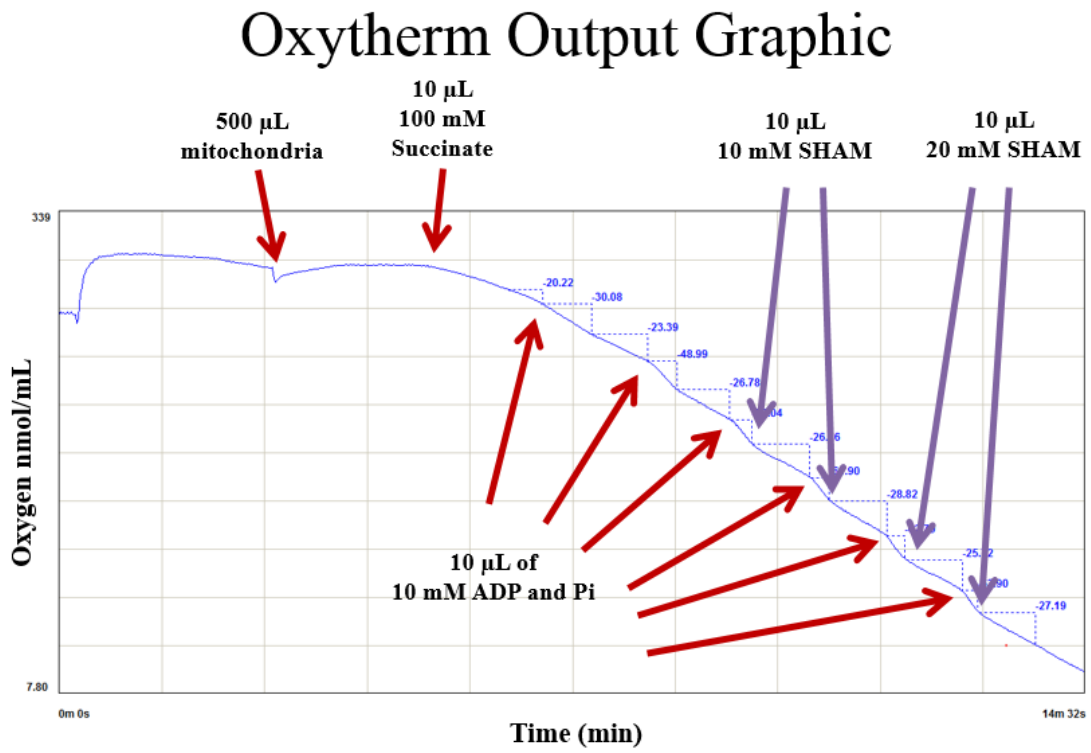

Figure 3 represents an output of mitochondria respiration from the Oxytherm system. Oxygen levels were measured in nmol per mL over the course of time in minutes. Reaction medium was added to provide a place for the reactions to occur. Mitochondria were added once the oxygen uptake rate had steadied from the addition of reaction medium. The addition of mitochondria caused an increase in the consumption of oxygen. This was state I respiration. Once the rate of oxygen uptake steadied, succinate was added to the system. Succinate was the source of electrons for this reaction. There was a slow decline in oxygen due to the consumption of oxygen to make water by the electron transport system (state II respiration). After the oxygen uptake rate steadied, ADP and P_i were added to the system. Addition of ADP and P_i allowed the ETC to rapidly produce ATP. This was state III respiration. Once the added ADP and P_i have been converted to ATP, the rate of oxygen consumption returned to a similar rate as before the addition (state IV respiration). At the end of the third cycle of phosphorylation, SHAM, which inhibits alternative oxidase, was added the system (Siedow & Umbach, 1995). Additions of ADP and P_i and SHAM were made until oxygen levels in the system were below the 0 calibration level (Gnaiger, 2010).

Respiration Rates and ADP:O Ratio Calculations

The oxytherm system calculated respiration rates. State III respiration rates used in the analysis represented the entire state III respiration cycle. State IV respiration rates used in the analysis represented the last 30 seconds of State IV respiration. ADP:O ratios were calculated by hand from the oxytherm output. To calculate ADP:O ratios, the number of nanomoles of oxygen at the beginning of state III respiration is multiplied by the milliliters of reaction medium and mitochondrial solution in the oxytherm chamber. This product was then multiplied by two (molecular oxygen). The same was done at the end of state III respiration. The product of the end of state 3 respiration calculation was subtracted from the product of the beginning of state III respiration calculation. The volume of ADP and P_i times the concentration of ADP and P_i is then divided by the difference of the state III respiration calculation (Eastabrook, 1967). There were a few problems with calculating ADP:O ratios. The transition from state 3 to state 4 respiration was not always obvious. This made calculating ADP:O ratios difficult because slight variations in the nanomoles of oxygen at the end of state III respiration can have a big impact on the ADP:O ratio. To avoid bias in calculations, two people calculated ADP:O ratios. No bias was found and my data were used.

AOX Ratios and Respiration Rate Ratios

To determine if the alternative oxidase inhibitor SHAM had an effect on ADP:O ratios and respiration rates, a ratio of each was calculated. The ADP:O ratio after the addition of SHAM over the ADP:O ratio before the addition of SHAM was termed the "AOX ratio." An AOX ratio greater than 1 would indicate that the ADP:O ratio after the addition of SHAM was larger than the ADP:O ratio before the addition of SHAM. The

respiration rate ratio represents the respiration rate after the addition of SHAM over the respiration rate before the addition of SHAM.

Protein Quantity Assay

DC™ Protein Assay from Bio Rad was used to determine the amount of mitochondria per experimental observation. Three replications of a standard curve were prepared to create a standard curve ranging from 1 mg/mL to 6 mg/mL of mitochondria. The following table was used to make the concentration gradients:

Table 1. The amounts of BSA and Suspension Medium required to make a 1-6 mg/mL concentration gradient

Concentration (mg/mL)	µL of 20 mg/mL BSA	µL Suspension Medium
1	25	475
2	50	450
3	75	425
4	100	400
5	125	375
6	150	350

One hundred µL of each level of the concentration gradient was added to the standard curve tubes and 100 µL of suspension medium was added to the "blank" tubes. One-hundred µL of mitochondrial sample were added to sample tubes. Five hundred µL of a combined solution of Reagent A and Reagent S were added to each tube. The amount of this solution was determined by counting the total number of blank, standard curve, and sample tubes. This number was rounded up to the nearest even number and divided by 2. This number was equivalent to the mL Reagent A needed. This number was multiplied by 20 to determine the µL of Reagent S needed. Once the 500 µL was added, each tube was vortexed. Four mL of Reagent B were added to each tube. Each tube was vortexed immediately after addition of Reagent B. If there was a color separation, the

tubes needed to be manually shaken. The tubes rested for 15 minutes. One mL of solution from each tube was added to spectrophotometer cuvettes. Absorbance was read at 750 nm. Standard curves were generated using y = (absorbance – b)/m. The standard curve with the highest R values was used for protein quantity determination.

Statistical Analysis

Both experiments (optimal temperature and low temperature stress) were arranged in a full factorial of a randomized complete block design with time as the blocking factor. There were four replications. Both hybrid and treatment were treated as fixed variables. Seedling mesocotyl lengths and total seedling lengths were measured for three sets of 25 seedlings for each experimental observation. Respiration rates were recorded and ADP:O ratios were calculated from the oxytherm output data. An analysis of variance was conducted by using PROC GLM in SAS 9.4. PROC MIXED was used to determine the differences in means, and PROC GLIMMIX were used to plot the means sliced by hybrid. A Tukey-Kramer adjustment was made. Differences of a p-value of less than 0.05 were considered significant.

CHAPTER THREE – RESULTS & DISCUSSION

Optimal Temperature Experiment

Mesocotyl and Total Seedling Length

Seedling mesocotyls from treated seeds were 0.41-0.48 cm longer than control (untreated) seedlings (Table 2). There was no difference in mesocotyl length between seedlings from seeds treated with Stamina and seedlings from seeds treated with only binders. There was no significant hybrid by treatment interaction. However, there were some significant differences in the differences of hybrid by treatment least squares means (Table 3). Seedling mesocotyl lengths of seed lot/hybrid B were the same for all treatments. Seedling mesocotyl lengths of seeds from seed lot/hybrid A treated with Stamina were longer than control seedlings from the same seed lot/hybrid. Seedlings from Stamina treated seeds of seed lot/hybrid A had longer mesocotyls than seedlings from Stamina treated seeds of seed lot/hybrid B (Figure 4). However the difference in mesocotyl length of Stamina and control seedlings for both hybrids is about the same (Table 3).

Total seedling lengths from Stamina treated seeds were 0.38 cm and 0.87 cm longer than the total seedlings lengths from seeds treated with only binders and control seedlings, respectively (Table 2). The total seedling length from seeds treated with only binders was greater than control seedlings. There was no significant hybrid by treatment interaction. However, there were some significant differences in the differences of hybrid by treatments least squares means (Table 3). Seedlings from seed lot/hybrid A treated with Stamina were longer than seedlings from the same seed lot/hybrid treated with only

binders and control seedlings. Seedlings from seed lot/hybrid B treated with Stamina and only binders were longer than control seedling of seed lot/hybrid B. Seed lot/hybrid A seedlings had longer total lengths than seed lot/hybrid B (Figure 5).

Strobilurins are believed to have positive physiological related effects on growth and yield (Gerhard et al., 1998; Grossmann & Retzlaff, 1997; Ishikawa et al., 2011). Grossman and Retzlaff (1997) conducted biotests on several species, including maize, to characterize the physiological mode of action of the strobilurin, kresoxim-methyl. The responses to kresoxim-methyl were similar to auxin responses.

Table 2. Optimal Temperature Maize mesocotyl and total seedling length measurements by treatment

Treatment	**Length (cm)** [A]	
	Mesocotyl[B]	Total[B]
Stamina	9.27a	13.17a
Binders Only	9.20a	12.79b
Control	8.79b	12.30c

[A] *Values represent the LS-means of four replications of approximately 75 seeds per replication averaged over hybrid*

[B] *Means within the same column followed by the same letter are not significantly different at P ≤ 0.05*

Table 3. Optimal Temperature Maize mesocotyl and total seedling length measurements by treatment and hybrid

Treatment		**Length (cm)** [A]	
	Hybrid	Mesocotyl[B]	Total[B]
Stamina	A	9.54a	14.04a
Binders Only	A	9.37ab	13.35b
Control	A	9.03bc	13.02b
Stamina	B	9.01bc	12.30c
Binders Only	B	9.03bc	12.23c
Control	B	8.54c	11.57d

[A] *Values represent the LS-means of four replications of approximately 75 seeds per replication*

[B] *Means within the same column followed by the same letter are not significantly different at P ≤ 0.05*

Optimal Temperature LS-Mean Mesocotyl Lengths by Treatment and Hybrid

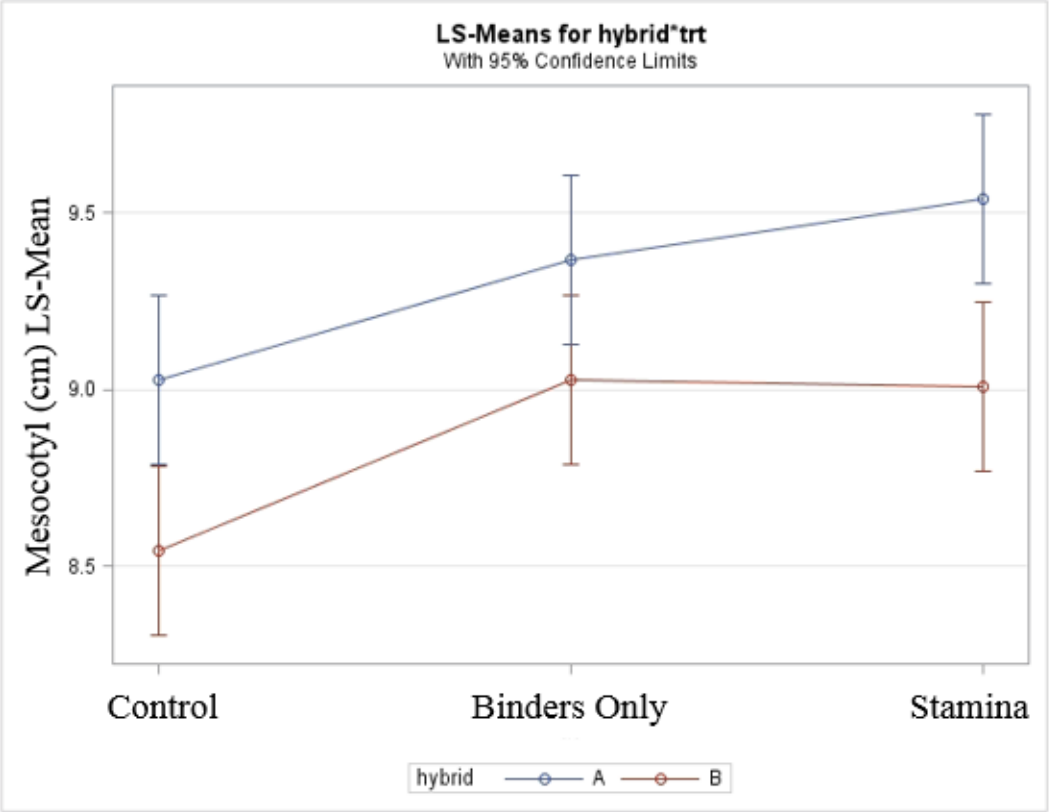

Figure 4: Optimal temperature study mesocotyl length LS means in centimeters by treatment, separated by hybrid with confidence intervals. Seed lot/hybrid A is on top (blue) and seed lot/hybrid B is on bottom (red). There was no significant treatment by hybrid interaction. However, the differences in LS means indicated a significant difference in mesocotyl length between the seed lot/hybrids for seedlings from seeds treated with Stamina.

Optimal Temperature LS-Means Total Seedling Length by Treatment and Hybrid

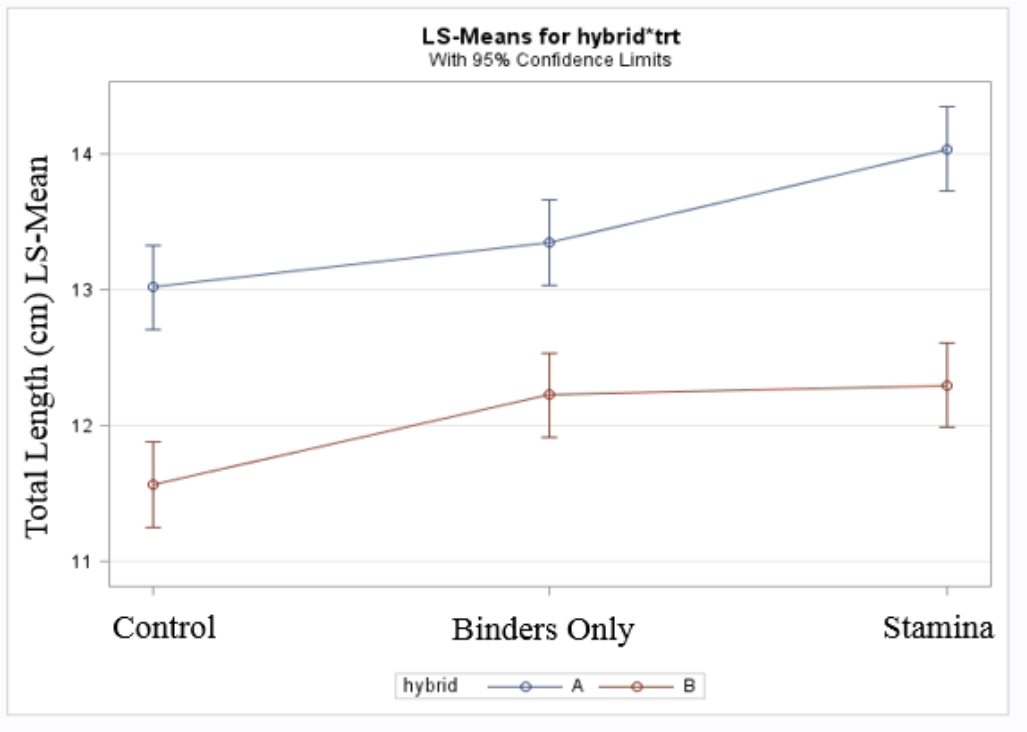

Figure 5: Optimal temperature study total length LS means in centimeters by treatment, separated by hybrid with confidence intervals. Seed lot/hybrid A is on top (blue) and seed lot/hybrid B is on bottom (red). Seed lot/hybrid A was significantly longer than seed lot/hybrid B for all treatments.

ADP:O Ratios

Stamina had no significant effect on ADP:O ratios before the addition of SHAM, ADP:O ratios after the addition of SHAM, or AOX ratios (Table 4 and Table 5).

Under optimal temperature conditions, electrons can be distributed between the cytochrome oxidase and alternative oxidase pathways (Ribas-Carbo et al., 1995). According to Prasad et al. (1994a), around 60% of O_2 uptake occurs via the cytochrome oxidase pathway and around 20% via alternative oxidase; indicating that even under optimal temperature conditions there is alternative oxidase activity (Stewart et al., 1990a). If alternative oxidase were up regulated, ADP:O ratios after the addition of

SHAM would be lower than the ADP:O ratios before the addition of SHAM (Noctor et al., 2007; Bendall & Bonner, 1971). A higher AOX ratio would be expected in seedlings with up-regulated alternative oxidase.

The lack of a difference in ADP:O ratios may be due to several factors. These treatments may not be involved in the up-regulation of alternative oxidase above normal levels for maize seedlings grown under optimal temperature condition. The use of a cytochrome oxidase inhibitor increases the sensitivity of SHAM (Prasad et al., 1994a). Because no cytochrome oxidase inhibitor was used, the concentration of SHAM may not have been enough to accurately measure alternative oxidase activity (Prasad et al., 1994a).

Table 4. Optimal Temperature Maize Mitochondria ADP:O Ratios by Treatment

Treatment	**ADP:O Ratios** [A]		
	Before SHAM[B]	After SHAM[B]	AOX Ratio [B]
Stamina	1.54a	1.54a	1.00a
Binders Only	1.43a	1.55a	1.10a
Control	1.47a	1.47a	1.00a

[A] Values represent the LS-mean ADP:O ratios before the addition and after the addition of SHAM averaged over hybrid

[B] Means within the same column followed by the same letter are not significantly different at $P \leq 0.05$

Table 5. Optimal Temperature Maize Mitochondria ADP:O Ratios by Treatment and Hybrid

Treatment		**ADP:O Ratios** [A]		
	Hybrid	Before SHAM[B]	After SHAM[B]	AOX Ratio [B]
Stamina	A	1.62a	1.63a	1.01a
Binders Only	A	1.47a	1.56a	1.08a
Control	A	1.50a	1.44a	0.97a
Stamina	B	1.46a	1.46a	1.00a
Binders Only	B	1.39a	1.55a	1.11a
Control	B	1.44a	1.49a	1.04a

[A] Values represent the LS-mean ADP:O ratios before the addition and after the addition of SHAM

[B] Means within the same column followed by the same letter are not significantly different at $P \leq 0.05$

Respiration Rates

Stamina had no significant effect on state III or state IV respiration rates before the addition of SHAM, after the addition of SHAM, or the ratio of respiration rates (Table 6, Table 7, Table 8, and Table 9). State IV respiration rates after the addition of SHAM tended to be lower than before the addition of SHAM (Table 8).

Under normal temperature conditions, electrons can be distributed between the cytochrome oxidase and alternative oxidase pathways (Ribas-Carbo et al., 1995). According to Prasad et al. (1994a), around 60% of O_2 uptake occurs via the cytochrome oxidase pathway and around 20% via alternative oxidase, indicating that even under optimal temperature conditions there is alternative oxidase activity (Stewart et al., 1990a). According to Dry et al. (1989), during state IV respiration the ubiquinone pool becomes more reduced, which can lead to higher levels of alternative oxidase. This could explain the general decrease in state IV respiration rates after the addition of SHAM.

The lack of a difference in respiration rates may be due to several factors. These treatments may not be involved in the up-regulation of alternative oxidase above normal levels for maize seedlings grown in optimal temperature conditions. The use of a cytochrome oxidase inhibitor increases the sensitivity of SHAM (Prasad et al., 1994a). Because no cytochrome oxidase inhibitor was used, the concentration of SHAM may not have been enough to accurately measure alternative oxidase activity (Prasad et al., 1994a).

Table 6. Optimal Temperature Maize Mitochondria State III Respiration Rates by Treatment

Treatment	**Respiration Rate (O_2 uptake nmol/mL/min)** [A]		
	State III Before[B]	State III After[B]	Ratio [B]
Stamina	62.05a	64.77a	1.05a
Binders Only	56.74a	58.56a	1.04a
Control	68.95a	68.12a	1.00a

[A] Values represent the LS-mean state III respiration rates before the addition and after the addition of SHAM averaged over hybrid

[B] Means within the same column followed by the same letter are not significantly different at $P \leq 0.05$

Table 7. Optimal Temperature Maize Mitochondria State III Respiration Rates by Treatment and Hybrid

Treatment		**Respiration Rate (O_2 uptake nmol/mL/min)** [A]		
	Hybrid	State III Before[B]	State III After[B]	Ratio [B]
Stamina	A	61.67a	66.73a	1.09a
Binders Only	A	55.22a	57.85a	1.06a
Control	A	68.09a	70.40a	1.04a
Stamina	B	62.43a	62.81a	1.01a
Binders Only	B	58.25a	59.26a	1.02a
Control	B	69.82a	65.83a	0.96a

[A] Values represent the LS-mean state III respiration rates before the addition and after the addition of SHAM

[B] Means within the same column followed by the same letter are not significantly different at $P \leq 0.05$

Table 8. Optimal Temperature Maize Mitochondria State IV Respiration Rates by Treatment

Treatment	**Respiration Rate (O_2 uptake nmol/mL/min)** [A]		
	State IV Before[B]	State IV After[B]	Ratio [B]
Stamina	27.54a	26.27a	0.96a
Binders Only	25.41a	24.42a	0.96a
Control	30.62a	29.60a	0.97a

[A] Values represent the LS-mean state IV respiration rates before the addition and after the addition of SHAM averaged over hybrid

[B] Means within the same column followed by the same letter are not significantly different at $P \leq 0.05$

Table 9. Optimal Temperature Maize mitochondria State IV Respiration Rates by Treatment and Hybrid

Treatment		**Respiration Rate (O_2 uptake nmol/mL/min)** [A]		
	Hybrid	State IV Before[B]	State IV After[B]	Ratio [B]
Stamina	A	27.44a	27.74a	1.01a
Binders Only	A	25.11a	24.70a	1.02a
Control	A	31.96a	30.93a	0.97a
Stamina	B	27.63a	24.80a	0.96a
Binders Only	B	25.72a	24.14a	0.93a
Control	B	29.28a	28.27a	0.90a

[A] Values represent the LS-mean state IV respiration rates before the addition and after the addition of SHAM

[B] Means within the same column followed by the same letter are not significantly different at $P \leq 0.05$

Low Temperature Stress Experiment

Mesocotyl and Total Seedling Length

Seedlings from Stamina treated seeds had longer mesocotyl lengths than seedlings from seeds treated with only binders and the control seedlings (Table 10). There was no difference in the mesocotyl length between the seedlings from seeds treated with only binders and the control seedlings. There was a hybrid by treatment interaction (Table 11). The mesocotyl lengths of seed lot/hybrid A were the same for all treatments. Seedlings from seeds treated with only binders and control seedlings of seed lot/hybrid A had longer mesocotyl lengths than seedlings from seed lot/hybrid B for the same treatments (Figure 6). Seedlings from seeds treated with Stamina of seed lot/hybrid B had mesocotyl lengths that were 0.9 cm longer than seedlings from seeds treated with only binders and control seedlings for the same seed lot/hybrid. Seedlings from seeds treated with Stamina of seed lot/hybrid B had the same mesocotyl length as seedlings from seeds treated with Stamina of seed lot/hybrid A.

Seedlings from Stamina treated seeds had longer total seedling lengths than seedlings from seeds treated with only binders and the control seedlings (Table 10). There was no difference in total seedling length between the seedlings from seeds treated with only binders and control seedlings. There was a hybrid by treatment interaction (Table 11). The total seedling lengths of seed lot/hybrid A were the same for all treatments and were also longer than the total seedlings lengths of seed lot/hybrid B for all treatments (Figure 7). Seedlings from seeds treated with Stamina of seed lot/hybrid B had total lengths that were 1.16 cm and 1.21 cm longer than seedlings from seeds treated with only binders and control seedlings of the same seed lot/hybrid.

The results of this study are similar to those of a study previously conducted in our lab (Hejlik, 2012). Low temperature stress can inhibit maize seedling germination and growth compared to maize seedling grown under optimal temperature conditions (Prasad et al., 1994a). According to Stewart et al. (1990a), seedlings that grow better under low temperature stress have higher levels of alternative oxidase activity; and seedlings that do not grow well under low temperature stress have lower levels of alternative oxidase.

In this stress temperature study Stamina had a greater impact on seed lot/hybrid B, even though seed lot/hybrid A tended to be longer. In the optimal temperature study, there was not a significant difference in the performance between the hybrids. The difference in hybrid performance in this study may be due to the tendency of seed treatments to improve growth potential, especially in lower quality seed lots (Munkvold, 2009). Gerhard et al. (1998) found that foliar applied kresoxim-methyl and azoxystrobin (strobilurins) impacted the leaf area of wheat cultivars differently depending on the environmental conditions. During a year of poor weather (drought) the leaf area of the stress susceptible cultivar was positively affected, but there was little difference in the leaf area of the less susceptible cultivar. Both cultivars performed similarly in a year without stress.

Strobilurins can also impact hormone levels (Grossmann & Retzlaff, 1997). According to Grossman & Retzlaff (1997), a foliar applied kresoxim-methyl decreased ACC Synthase levels and increased some cytokinin concentrations in wheat. ACC Synthase is an important enzyme in the synthesis of ethylene. Environmental stresses and developmental processes can induce higher levels of ethylene production. A decrease in

ACC Synthase leads to less ethylene production. Reducing ethylene production during stress could lead to improved growth and development (Grossmann & Retzlaff, 1997).

Table 10. Low Temperature Stress Maize Mesocotyl and Total Seedling Length Measurements by Treatment

Treatment	**Length (cm)** [A]	
	Mesocotyl[B]	Total[B]
Stamina	8.97a	12.49a
Binders Only	8.59b	12.04b
Control	8.46b	11.96b

[A] *Values represent the mean of four replications of approximately 75 seeds per replication averaged over hybrid*

[B] *Means within the same column followed by the same letter are not significantly different at P ≤ 0.05*

Table 11. Low Temperature Stress Maize Mesocotyl and Total Seedling Length Measurements by Treatment and Hybrid

Treatment		**Length (cm)** [A]	
	Hybrid	Mesocotyl[B]	Total[B]
Stamina	A	9.17a	13.01a
Binders Only	A	9.31a	13.27a
Control	A	9.08a	13.15a
Stamina	B	8.76a	11.97b
Binders Only	B	7.87b	10.81c
Control	B	7.85b	10.76c

[A] *Values represent the mean of four replications of approximately 75 seeds per replication averaged over hybrid*

[B] *Means within the same column followed by the same letter are not significantly different at P ≤ 0.05*

Low Temperature Stress LS-Mean Seedling Mesocotyl Length by Treatment and Hybrid

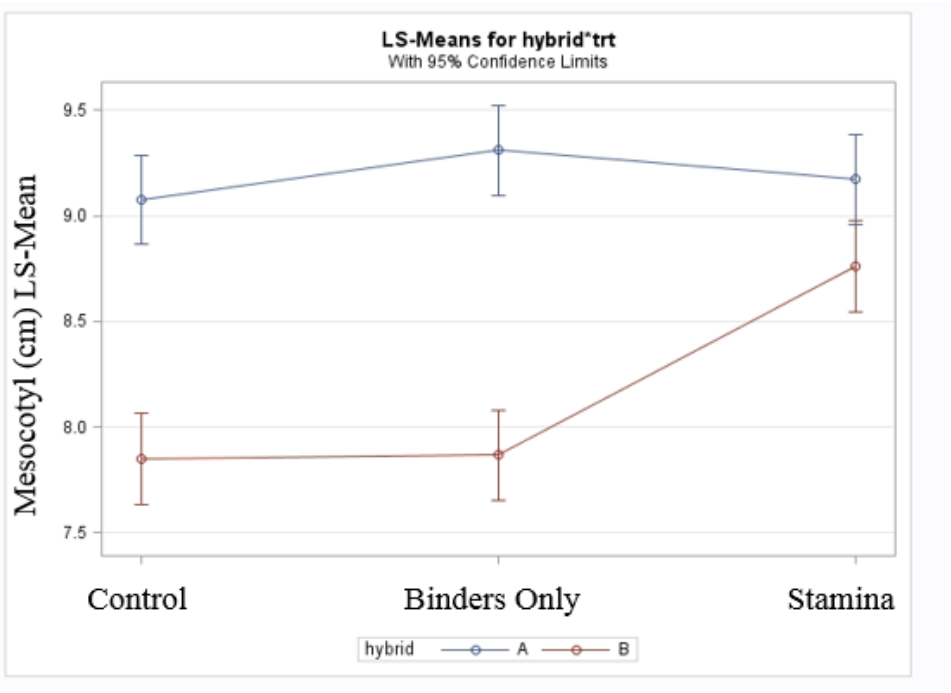

Figure 6: Low temperature stress study mesocotyl length LS means in centimeters by treatment, separated by hybrid with confidence intervals. Seed lot/hybrid A is on top (blue) and seed lot/hybrid B is on bottom (red). There was a significant hybrid by treatment interaction. Seed lot/hybrid A had longer mesocotyls than seed lot/hybrid B for control and binders only treatments. Seedlings from seeds treated with Stamina of seed lot/hybrid B had longer mesocotyl lengths than seedlings from seeds treated with only binders and control seedlings. Seedlings from Stamina treated seeds of seed lot/hybrid B had the same mesocotyl lengths had seedlings from Stamina treated seeds of seed lot/hybrid A.

Low Temperature Stress LS-Mean Total Seedling Length by Treatment and Hybrid

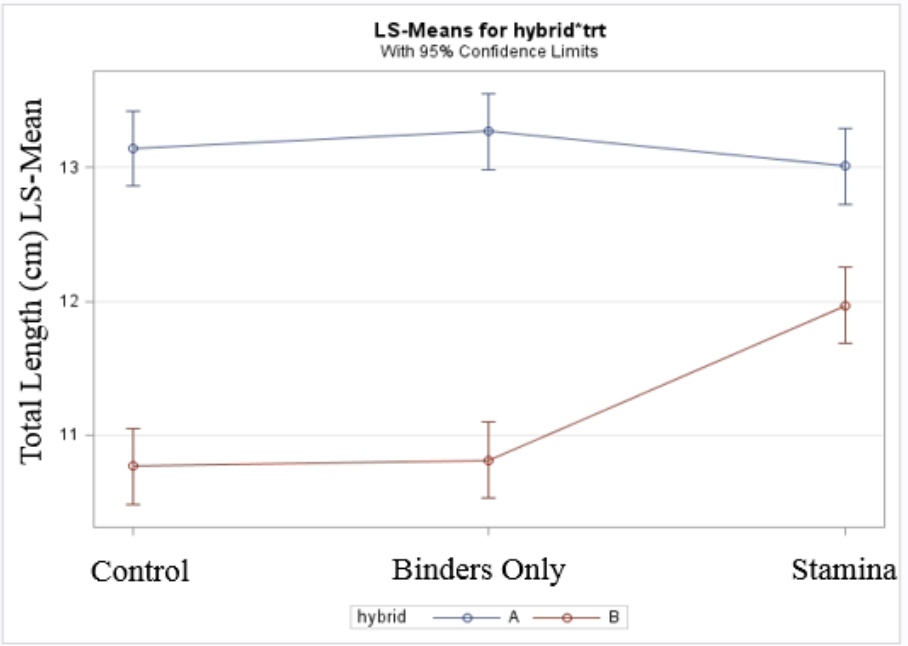

Figure 7: Low temperature stress study total length LS means in centimeters by treatment, separated by hybrid with confidence intervals. Seed lot/hybrid A is on top (blue) and seed lot/hybrid B is on bottom (red). Seed lot/hybrid A was significantly longer than seed lot/hybrid B for all treatments. Seedlings from Stamina treated seeds of seed lot/hybrid B had longer total lengths than seedlings from seeds treated with only binders and control seedlings of seed lot/hybrid B.

ADP:O Ratios

Stamina had no effect on ADP:O ratios or AOX ratios when 10 mM of SHAM was used. Seedlings from seeds treated with Stamina and control seedlings had larger AOX ratios than seedling from seeds treated with only binders when 20 mM of SHAM was used (Table 12). There was no significant hybrid by treatment interaction. However, there were some differences in the differences of hybrid by treatment least squared means (Table 13). Seedlings from Stamina treated seeds and control seedlings had larger AOX ratios than seeds treated with only binder in seed lot/hybrid B. This difference was not see in seed lot/hybrid A.

According to Stewart et al. (1990a), low temperature stress can up regulate alternative oxidase in maize seedlings, leading to lower ADP:O ratios (Noctor et al., 2007). Inhibiting alterative oxidase can force electrons through the cytochrome oxidase pathway, resulting in higher ADP:O ratios (Hoefnagel et al., 1995). If alternative oxidase is up regulated, ADP:O ratios would be higher in the presence of SHAM. If Stamina was also involved in the up regulation of alternative oxidase, ADP:O ratios before the addition of SHAM would be lower compared to other treatments. A lower ADP:O ratio before the addition of SHAM would also lead to a larger AOX ratio. This was only the case for seedlings from Stamina treated seeds and control seedlings of seed lot/hybrid B.

Several factors could affect these results. It has been demonstrated that the alternative oxidase activity is up regulated under low temperature conditions but can be rapidly lost within a few days of returning to normal conditions (Elthon et al., 1986; Prasad et al., 1994a; Stewart et al., 1990a, 1990b). The recovery period for this study may have been too long of a time frame to measure increased levels of alternative oxidase. It is also possible that without a cytochrome oxidase inhibitor, the concentration of SHAM was not enough to accurately determine the level of alternative oxidase (Prasad et al., 1994a). However, the concentrations used should have been enough to inhibit at least some alternative oxidase activity, especially with 20 mM SHAM (Spreen Brouwer et al., 1986). In practice, 10 mM SHAM was enough to see a difference in ADP:O ratios under low temperature stress.

Table 12. Low Temperature Stress Maize Mitochondria ADP:O Ratios by Treatment

Treatment	**ADP:O Ratios** [A]				
	Before[B]	10mM SHAM[B]	10mM AOX Ratio [B]	20mM SHAM [B]	20mM AOX Ratio [B]
Stamina	1.23a	1.33a	1.08a	1.41a	1.15a
Binders Only	1.26a	1.31a	1.04a	1.34a	1.07b
Control	1.20a	1.29a	1.08a	1.38a	1.15a

[A] Values represent the LS-mean ADP:O ratios before the addition and after the addition of SHAM averaged over hybrid

[B] Means within the same column followed by the same letter are not significantly different at $P \leq 0.05$

Table 13. Low Temperature Maize Mitochondria ADP:O Ratios by Treatment and Hybrid

Treatment		**ADP:O Ratios** [A]				
	Hybrid	Before[B]	10mM SHAM[B]	10mM AOX Ratio [B]	20mM SHAM [B]	20mM AOX Ratio [B]
Stamina	A	1.20a	1.26a	1.05a	1.35a	1.12ab
Binders Only	A	1.18a	1.27a	1.07a	1.31a	1.10ab
Control	A	1.15a	1.24a	1.08a	1.29a	1.13ab
Stamina	B	1.25a	1.39a	1.11a	1.47a	1.17a
Binders Only	B	1.33a	1.35a	1.01a	1.38a	1.03b
Control	B	1.25a	1.34a	1.08a	1.46a	1.17a

[A] Values represent the LS-mean ADP:O ratios before the addition and after the addition of SHAM

[B] Means within the same column followed by the same letter are not significantly different at $P \leq 0.05$

Respiration Rates

Stamina did not have a significant effect on the state III respiration rates before or after the addition of 10 mM and 20 mM SHAM. Seedlings from Stamina treated seeds had larger respiration rate ratios than seedlings from seeds treated with only binders with the addition of 10 mM SHAM (Table 14). However, the respiration rate ratio for the seedling from Stamina treated seeds was slightly larger than 1, indicating that the respiration rate after the addition of SHAM was larger than before the addition of SHAM. The respiration rate ratio for the seedling from seeds treated with only binders was slightly lower than 1, indicating that the respiration rate after the addition of SHAM was lower than before the addition of SHAM.

There was a hybrid by treatment interaction (Table 15). For seed lot/hybrid A, seedlings from Stamina treated seeds and control seedlings had respiration rate ratios

slightly above 1. Seedlings from seeds treated with only binders had a respiration rate ratio slightly below 1. For seed lot/hybrid B, seedlings from Stamina treated seeds had a larger respiration rate ratio than the control seedlings. The respiration rate for the seedlings from Stamina treated seeds had a ratio slightly above 1 and control seedlings had a ratio slightly below 1. Although significant, these results may be questionable due to a lack of consistency among treatments and hybrids.

Stamina did not have a significant effect on the state IV respiration rates before the addition of SHAM, after the addition of SHAM, or the respiration rate ratio (Tables 16 & 17). State IV respiration rates were lower after the addition of 20 mM of SHAM compared to 10 mM SHAM (Table 17).

According to Prasad et al. (1994a) respiration rates are lower under low temperature stress conditions. This may be due to damage done to the ETC, decreased ATP synthase activity, and electron flow through alternative oxidase (Prasad et al., 1994a). Prasad et al. (1994a) found that under low temperature stress, O_2 uptake by the cytochrome oxidase pathway decreased from around 60% to around 30% mitochondria respiration and O_2 uptake by alternative oxidase increased from around 20% to around 37% mitochondria respiration. However, after only a few days of recovery time, O_2 uptake by the cytochrome pathway returned to previous levels in seedlings that survived the low temperature stress (Prasad et al., 1994a; Stewart et al., 1990b). According to Dry et al. (1989), during state IV respiration the ubiquinone pool becomes more reduced, which can lead to higher levels of alternative oxidase. This could explain the general decrease in state IV respiration rates after the addition of SHAM.

Several factors could affect these results. Alternative oxidase activity is upregulated under low temperature conditions but can be rapidly lost within a few day of returning to normal conditions (Elthon et al., 1986; Prasad et al., 1994a; Stewart et al., 1990b). The recovery period for this study may have been too long of a time frame to measure increased levels of alternative oxidase. It is also possible that without a cytochrome oxidase inhibitor, the concentration of SHAM was not enough to accurately measure alternative oxidase activity (Prasad et al., 1994a). However, the concentrations used should have been enough to inhibit at least some alternative oxidase activity, especially with 20 mM (Spreen Brouwer et al., 1986). In practice, 10 mM SHAM was enough to see a difference in respiration rates under low temperature stress.

Table 14. Low Temperature Stress Maize Mitochondria State III Respiration Rates by Treatment

Treatment	**Respiration Rate (O_2 uptake nmol/mL/min)** [A]				
	Before[B]	10 mM SHAM[B]	10 mM Ratio [B]	20 mM SHAM [B]	20 mM Ratio [B]
Stamina	64.72a	66.95a	1.03a	65.45a	1.02a
Binders Only	72.70a	69.33a	0.96b	68.04a	0.94a
Control	76.46a	74.68a	0.99ab	73.04a	0.96a

[A] Values represent the LS-mean state III respiration rates before the addition and after the addition of SHAM averaged over hybrid

[B] Means within the same column followed by the same letter are not significantly different at $P \leq 0.05$

Table 15. Low Temperature Stress Maize mitochondria State III Respiration Rates by Treatment and Hybrid

Treatment		**Respiration Rate (O_2 uptake nmol/mL/min)** [A]				
	Hybrid	Before[B]	10 mM SHAM[B]	10 mM Ratio [B]	20mM SHAM [B]	20mM Ratio [B]
Stamina	A	65.28a	67.86a	1.04a	65.75a	1.01a
Binders Only	A	76.36a	70.94a	0.93b	71.35a	0.94a
Control	A	73.88a	77.48a	1.05a	75.34a	1.03a
Stamina	B	64.16a	66.05a	1.03a	65.15a	1.02a
Binders Only	B	69.05a	67.72a	1.00ab	64.73a	0.93a
Control	B	79.03a	72.26a	0.92b	70.74a	0.90a

[A] Values represent the LS-mean state III respiration rates before the addition and after the addition of SHAM

[B] Means within the same column followed by the same letter are not significantly different at $P \leq 0.05$

Table 16. Low Temperature Stress Maize Mitochondria State IV Respiration Rates by Treatment

Treatment	**Respiration Rate (O_2 uptake nmol/mL/min)** [A]				
	Before[B]	10mM SHAM[B]	10mM Ratio [B]	20mM SHAM [B]	20mM Ratio [B]
Stamina	29.44a	28.18a	0.96a	25.35a	0.86a
Binders Only	33.13a	31.13a	0.94a	27.95a	0.84a
Control	35.01a	33.56a	0.96a	30.12a	0.87a

[A] *Values represent the LS-mean state IV respiration rates before the addition and after the addition of SHAM averaged over hybrid*

[B] *Means within the same column followed by the same letter are not significantly different at $P \leq 0.05$*

Table 17. Low Temperature Stress Maize mitochondria State IV Respiration Rates by Treatment and Hybrid

Treatment		**Respiration Rate (O_2 uptake nmol/mL/min)** [A]				
	Hybrid	Before[B]	10mM SHAM[B]	10mM Ratio[B]	20mM SHAM [B]	20mM Ratio [B]
Stamina	A	28.51a	27.55a	0.97a	25.04a	0.88a
Binders Only	A	35.32a	32.97a	0.94a	29.46a	0.84a
Control	A	34.50a	34.07a	1.00a	30.56a	0.90a
Stamina	B	30.38a	33.06a	0.95a	25.67a	0.84a
Binders Only	B	30.95a	29.28a	0.94a	26.45a	0.84a
Control	B	35.15a	38.80a	0.93a	29.68a	0.84a

[A] *Values represent the LS-mean state IV respiration rates before the addition and after the addition of SHAM*

[B] *Means within the same column followed by the same letter are not significantly different at $P \leq 0.05$*

CHAPTER FIVE – CONCLUSIONS

Stamina is a strobilurin fungicide used primarily in maize and soybeans (Munkvold, 2009). Although Stamina is primarily used to protect against fungal pathogens, it is believed to have several positive physiological related effects (Gerhard et al., 1998; Grossmann & Retzlaff, 1997; Ishikawa et al., 2011). The goal of this study was to determine the effects on Stamina on seedling length, ADP:O ratios, and respiration rates, and alternative oxidase amounts (appendix) under optimal temperatures and low temperature stress conditions in the absence of fungal pathogens.

Stamina did have an impact on mesocotyl and total seedling length. Under optimal temperature conditions, the seedlings from seeds treated with Stamina and only binders had longer mesocotyls than the untreated seeds. Seedlings from Stamina treated seeds had longer total lengths. Under low temperature stress conditions, Stamina had a greater impact on seed lot/hybrid B. Seedlings from seeds treated with Stamina of seed lot/hybrid B treated had longer mesocotyl and total lengths than seedlings from seeds treated with only binders and control seedlings. There was no visible evidence to suggest this difference in seed lot/hybrid performance was pathogen related. This difference in performance could be due to differences in seed lot quality or genetics. If there was a difference in seed lot quality, there may have been differences in respiration rates (Woodstock & Grabe, 1967). However, there were no observed respiration rate differences between seed lot/hybrids.

There was no clear evidence from this study that Stamina had an impact on alternative oxidase activity. There were a few differences in respiration rate ratios and

AOX ratios under low temperature stress. However, these results may be questionable due to a lack of consistency among treatments and hybrids.

More research is needed in the lab and field before all physiological impacts of Stamina are understood. The use of a cytochrome inhibitor, a shorter temperature recovery period, more seed lots/hybrids, and AOX gene probing may aid in future research to obtain a clearer picture of the physiological impacts of Stamina.

APPENDIX – ALTERNATIVE OXIDASE AMOUNT

Plant Alternative Oxidase Elisa Assay

Alternative oxidase protein quantities in mitochondria samples were assayed with a Plant Alternative Oxidase (AOX) Elisa kit (SunRed Biological Technology). This kit uses an anti-synthetic polypeptide antibody. This antibody was compared with the monoclonal antibody developed by Elthon et al. (1989) and found to be effective (Ying-Cai et al., 2002). Mitochondrial samples were placed in the -80 °C freezer after use in the oxytherm until they were used in the elisa kit. Samples were thawed in the refrigerator (~5°C) and placed on ice. Each sample was diluted to 1 mg/mL with suspension medium. To release the contents of the mitochondria, 300 uL of the a Big Chap detergent solution (1 mM EDTA, 30 mM TES, 0.5% Big Chap, Elthon & McIntosh 1986) were added to each sample tube to have a final concentration of 166.67 ug mitochondria per mL. Sample tubes were then sonicated twice for 5 seconds each on a setting of 3 on a Fisher Scientific 550 Sonic Dismembrator sonicator to ensure protein release (Dr. Thomas Elthon, personal communication, June 2014. Dr. Larry Halverson, personal communication, June 2014).

Samples were then assayed in accordance with the instructions provided with the kits. Samples from both optimal temperature and low temperature stress studies were randomly placed on two elisa plates. Elisa plates were read at both 405 nm (Trisha Scott, personal communication, 2014.) and 450 nm. Absorbance readings at 405 nm more accurately reflected the visible color changes on the plate and produced better standard curves. This data was used for the analysis. However, most data points fell below the standard curve of 5-80 ng/mL.

Optimal Temperature Study

Stamina did not effect on the amount of alternative oxidase protein in the maize mesocotyl mitochondria (Table 18 and Table 19).

According to Stewart et al. (1990a), maize seedlings have alternative oxidase activity at all temperatures. When the cytochrome oxidase has been inhibited in some manner, there is an increase in the alternative oxidase protein and activity (Prasad et al., 1994a). If Stamina blocked electron flow through cytochrome oxidase, an increase in the amount of alternative oxidase protein in seedlings from Stamina treated seeds would be expected. However, no increase in alternative oxidase was found in seedlings from Stamina treated seeds.

The lack of a significant difference among treatments may be due to several factors. Seedlings were grown under normal temperature conditions. If Stamina is not involved in the up-regulation of alternative oxidase activity, differences between treatments would not be expected. The quality of the kits may have also been a contributing factor.

Table 18. Optimal Temperature Maize Seedling Alternative Oxidase Protein by Treatment

Treatment	**Alternative Oxidase (ng/mL)** ^{AB}
Stamina	2.62a
Binders Only	2.79a
Control	2.86a

[A] Values represent the LS-mean amount of alternative oxidase protein averaged over hybrid

[B] Means within the same column followed by the same letter are not significantly different at $P \leq 0.05$

Table 19. Optimal Temperature Maize Seedling Alternative Oxidase Protein by Treatment and Hybrid

Treatment		**Alternative Oxidase (ng/mL)** [AB]
	Hybrid	
Stamina	A	2.70a
Binders Only	A	2.81a
Control	A	2.50a
Stamina	B	2.55a
Binders Only	B	2.76a
Control	B	3.23a

[A] *Values represent the LS-mean amount of alternative oxidase protein*

[B] *Means within the same column followed by the same letter are not significantly different at* $P \leq 0.05$

Low Temperature Stress Study

Stamina did not have an effect on the amount of alternative oxidase protein in the maize mesocotyl mitochondria (Table 20 and Table 21).

According to Stewart et al (1990a), there is an increase in alternative oxidase protein under low temperature stress; and maize seedlings that grow better under low temperature stress have higher levels of alternative oxidase. However, no differences in alternative oxidase protein amounts were found.

The lack of a significant difference among treatments may be due to several factors. All treatments were exposed to the same low temperature stress. If Stamina were involved in the up-regulation of alternative oxidase it would be expected that Stamina treated seedling would have the largest amounts of alternative oxidase protein. A four day temperature recovery time period may have been too long for continued expression of alternative oxidase (Elthon et al., 1986; Prasad et al., 1994a; Stewart et al., 1990b). The quality of the kits may have also been a contributing factor.

Table 20. Low Temperature Stress Maize Seedling Alternative Oxidase Protein by Treatment

Treatment	**Alternative Oxidase (ng/mL)** ^{AB}
Stamina	2.10a
Binders Only	1.95a
Control	2.40a

[A] Values represent the LS-mean amount of alternative oxidase protein averaged over hybrid

[B] Means within the same column followed by the same letter are not significantly different at P ≤ 0.05

Table 21. Low Temperature Stress Maize Seedling Alternative Oxidase Protein by Treatment and Hybrid

Treatment		**Alternative Oxidase (ng/mL)** ^{AB}
	Hybrid	
Stamina	A	2.37a
Binders Only	A	2.05a
Control	A	2.24a
Stamina	B	1.84a
Binders Only	B	1.86a
Control	B	2.56a

[A] Values represent the LS-mean amount of alternative oxidase protein

[B] Means within the same column followed by the same letter are not significantly different at P ≤ 0.05

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